Altered Expression of Cell Cycle Proteins and Prolonged Duration of Cardiac Myocyte Hyperplasia in p27\(\text{KIP1}\) Knockout Mice

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Abstract—The precise role of cell cycle–dependent molecules in controlling the switch from cardiac myocyte hyperplasia to hypertrophy remains to be determined. We report that loss of p27\(\text{KIP1}\) in the mouse results in a significant increase in heart size and in the total number of cardiac myocytes. In comparison to p27\(\text{KIP1}^{+/+}\) myocytes, the percentage of neonatal p27\(\text{KIP1}^{-/-}\) myocytes in S phase was increased significantly, concomitant with a significant decrease in the percentage of G\(_0\)/G\(_1\) cells. The expressions of proliferating cell nuclear antigen, G\(_1\)/S and G\(_2\)/M phase–acting cyclins, and cyclin-dependent kinases (CDKs) were upregulated significantly in ventricular tissue obtained from early neonatal p27\(\text{KIP1}^{-/-}\) mice, concomitant with a substantial decrease in the expressions of G\(_1\) phase–acting cyclins and CDKs. Furthermore, mRNA expressions of the embryonic genes atrial natriuretic factor and \(\alpha\)-skeletal actin were detectable at significant levels in neonatal and adult p27\(\text{KIP1}^{-/-}\) mouse hearts but were undetectable in p27\(\text{KIP1}^{+/+}\) hearts. In addition, loss of p27\(\text{KIP1}\) was not compensated for by the upregulation of other CDK inhibitors. Thus, the loss of p27\(\text{KIP1}\) results in prolonged proliferation of the mouse cardiac myocyte and perturbation of myocyte hypertrophy. (Circ Res. 1999;85:117-127.)

Key Words: cardiac myocyte • cell cycle • p27\(\text{KIP1}\) • hyperplasia • mouse

Terminal differentiation of the cardiac myocyte occurs at, or shortly after, birth in most mammals\(^1–3\) and is characterized by the transition from hyperplastic growth (cell division) to hypertrophic growth (an increase in cell size). Recently, Soonpaa et al\(^3\) have shown that, in the mouse heart, cardiomyocyte reduplication is completed by birth, with DNA synthesis in neonatal cells contributing only to binucleation. The cessation of myocyte proliferation has been attributed to a block in the cell cycle\(^4–6\) and in accordance with this hypothesis we recently have shown that adult rat cardiac myocytes display a dual cell cycle blockade, with >80% of cells arresting in G\(_0\)/G\(_1\) and >15% of cells arresting in G\(_2\)/M.\(^6–9\)

Cell cycle progression is known to be regulated both positively, through the formation of cyclin and cyclin-dependent kinase (CDK) complexes, and negatively, by cyclin-dependent kinase inhibitors (CDKIs), that bind to and inhibit the activities of various cyclin-CDK complexes (see References 5 and 6 for reviews). Currently, the mechanisms that regulate the cessation of cardiac myocyte hyperplasia remain unknown, although we and others recently have shown that certain cyclins, CDKs,\(^10–17\) and CDKIs\(^17–21\) may be involved. Indeed, the CDKI molecules p21\(\text{CIP1}\) and p27\(\text{KIP1}\) appear to play pivotal roles in the development of cardiac myocyte hypertrophy.\(^22\)

Recently, three independent groups have reported the generation of a mouse lacking the gene for p27\(\text{KIP1}\).\(^23–25\) p27\(\text{KIP1}\) is a member of the CIP/KIP family of CDKI molecules, which also contains the inhibitors p21\(\text{CIP1}\) and p57\(\text{KIP2}\).\(^5,6\) In contrast to the p21\(\text{CIP1}\) knockout mouse, which develops normally and shows no obvious changes in phenotype from the wild-type mouse,\(^26\) the p27\(\text{KIP1}\) knockout mouse exhibits enhanced growth and multiple organ enlargement and develops pituitary tumors.\(^23–25\) The enhanced organ size was shown to be due to an increase in cell number in those tissues studied, with no significant increase in cell size.\(^23–25\) Thus, the increase in cell number coupled with the development of pituitary tumors in these mice suggests that p27\(\text{KIP1}\) acts to regulate the cell division process in multiple cell types.

In an attempt to determine whether p27\(\text{KIP1}\) is involved in controlling cardiac myocyte hyperplasia and therefore regulates myocyte cell division, we have examined the cell cycle profiles of cardiac myocytes isolated from the hearts of p27\(\text{KIP1}^{+/+}\), p27\(\text{KIP1}^{-/-}\), and p27\(\text{KIP1}^{+/-}\) mice by flow cytometry and have determined the effects of the loss of p27\(\text{KIP1}\) on myocyte hyperplasia by immunocytochemistry of
whole ventricular sections. In addition, we have compared the expressions of certain cyclins, CDKs, CDKIs, the proliferation marker, proliferating cell nuclear antigen (PCNA), and the embryonic genes atrial natriuretic factor (ANF) and α-skeletal actin in whole ventricular tissues isolated from both p27\textsuperscript{KIP1}+/+/ wild-type and p27\textsuperscript{KIP1}−/− mice.

**Materials and Methods**

Antimouse immunoglobulin (Ig) G HRP-conjugated antibody, biotin-conjugated goat anti-rabbit IgG, streptavidin–Texas Red, and enhanced chemiluminescence Western blotting reagents were purchased from Amersham International PLC. DMEM with glutamax, M199 medium, pancreatin, and penicillin/streptomycin were obtained from Gibco. FCS was obtained from Globepharm. Collagenase CLS1 was purchased from Worthington. BSA (type V), pepsin, bromodeoxyuridine (BrdU), propidium iodide (PI), rabbit anti-goat FITC-conjugated IgG, rabbit anti-laminin polyclonal antibody, and ribonuclease A were purchased from Sigma. Rabbit polyclonal antibodies directed against p21CIP1, p27 KIP1, CDK2, CDK4, CDK6, cyclins A, E, D2, and D3, mouse monoclonal antibody against PCNA, and anti-rabbit IgG HRP-conjugated antibody were obtained from Santa Cruz Biotechnology Ltd. CDC2 rabbit polyclonal antibody was obtained from TCS. Anti-BrDU mouse monoclonal antibody was purchased from Becton Dickinson, and anti-mouse FITC-labeled antibody was purchased from Dako. A rabbit polyclonal antibody against cardiac-specific troponin I was a gift from Dr P. Cummins, University of Birmingham, UK. The avidin/biotin blocking kit was purchased from Vector Labs, and the cDNA reverse transcriptase–polymerase chain reaction (RT-PCR) kit was purchased from Invitrogen. The DNA restriction enzyme BglII and buffer 7 were purchased from Northumbria Biologicals Ltd. All other chemicals used were of the highest grade available commercially.

**Animals**

Neonatal control (p27\textsuperscript{KIP1}+/+) C57 Black mice aged 1, 2, 3, 4, and 21 days and adult (36 days) mice were obtained from Bentogen and Kingman, UK (n=3). p27\textsuperscript{KIP1} knockout mice at the same ages were bred and housed in the animal facility of the University College of London (n=3). Founder mice were supplied by Prof James Roberts, Fred Hutchinson Cancer Research Center, Seattle, Wash. Animals were killed by an approved method, in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986.

**Genotype Analysis of p27\textsuperscript{KIP1} Knockout Mice**

To determine the genotype of p27\textsuperscript{KIP1} knockout mice, the heart and lung tissues of each mouse were analyzed by a combination of protein immunoblotting and RT-PCR with an anti-p27 KIP1 antibody and p27\textsuperscript{KIP1} primers, respectively, exactly as described below. The genotype of each mouse was confirmed by an independent investigator located at a different research facility (Dr B. Durand, University College London [personal communication] and Reference 27) using immunoblotting of protein obtained from brain tissue from the same mouse. To verify the results obtained with protein immunoblotting, RT-PCR was performed and in each case gave an identical genotype to that identified by immunoblotting for each mouse.

**Cell Culture**

Murine NIH3T3 fibroblasts were maintained in DMEM containing 10% FCS in a humidified atmosphere containing 5% CO\textsubscript{2}/95% air at 37°C. Cells were rendered quiescent by placing in DMEM supplemented with 0.2% FCS for 48 hours before use. For serum-stimulated cells, quiescent fibroblasts were stimulated with 10% FCS for 72 hours.

**Cell Isolation**

Neonatal myocytes were isolated from individual p27\textsuperscript{KIP1}+/+ and p27\textsuperscript{KIP1} knockout mice according to methods described previously. Briefly, excised hearts were separated into ventricular and atrial tissues, and the ventricles were dissociated by serial enzymatic digestion (5 digestions of 7 minutes each) with 33 mg of collagenase CLS1 and 30 mg of pancreatin in 60 mL of 1× Ads buffer, using a shaking water bath at 37°C. Myocytes and nonmyocytes were separated by preplating for 30 minutes onto 10 cm² Primaria tissue culture plates (Falcon) in medium containing 68% DMEM-glutamax, 17% M199, 15% FCS, and 100 μg/mL penicillin/streptomycin. Isolated cells were used immediately. This method is used routinely in our laboratory and results in preparations containing >97% to 99% cardiac myocytes, as determined by light microscopy and immunostaining with a cardiac-specific troponin I antibody.

**BrdU Incorporation and Flow Cytometric Analysis**

Freshly isolated cardiac myocytes from p27\textsuperscript{KIP1}+/+ and p27\textsuperscript{KIP1} knockout mice aged 1, 2, 3, 4, and 6 days were prepared as described above. A total of 1×10\textsuperscript{6} myocytes were used for each sample, and BrdU incorporation and flow cytometric analyses were performed as described previously. Labeled nuclei were analyzed for BrdU incorporation and PI staining using a fluorescence-activated cell sorter (FACS) (Calibur) analyzer (Becton Dickinson). Myocyte nuclei were gated into the respective cell cycle phases (G0/G1, S, and G2/M phases) by analyzing BrdU versus PI plots using CellQuest software (Becton Dickinson).

**Protein Extraction and Immunoblotting**

Protein samples were prepared from lung and whole ventricular tissues obtained from p27\textsuperscript{KIP1}+/+ and p27\textsuperscript{KIP1} knockout mice aged 1, 2, 3, 4, and 6 days and adult mice. Samples were prepared according to methods described previously. Proteins were separated electrophoretically on either 12% or 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, 0.45 μm, Millipore) according to established methods. Membranes were incubated with rabbit polyclonal antibodies (1:1000 dilution) directed against various cyclins, CDKs, CDKIs, and PCNA, and immunoreactive proteins were detected according to methods described previously.

**Total RNA Preparation and RT-PCR**

Total RNA from whole ventricular tissue of p27\textsuperscript{KIP1}+/+ and p27\textsuperscript{KIP1} knockout mice aged 6 days and adult mice was prepared using RNA STAT-60 (Tel-Test B, Inc) exactly as recommended by the manufacturer. Ten micrograms of total RNA from each sample was reverse-transcribed using a cDNA RT-PCR kit as recommended by the manufacturer. After ethanol precipitation, cDNA samples were dissolved in 50 μL of double-distilled RNase-free water. One microliter of this diluted cDNA was used for PCR amplification of GAPDH, ANF, skeletal actin, and p27\textsuperscript{KIP1}. PCR was performed using the aforementioned 1 μL of diluted cDNA in a total volume of 50 μL containing 50 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl\textsubscript{2}, 100 μmol/L of each dNTP, 2.5 U Taq polymerase, and 100 pmol each of sense and antisense primers. Primers were directed against the mouse sequence for p27\textsuperscript{KIP1} (sense 5′-GCTTGAGGGCTGCGTACGGC-3′; antisense 5′-CTGTGAGGGCTGCGTACGGC-3′), GAPDH (sense 5′-ATGGGCTCCTTCTCCATCAC-3′; antisense 5′-TTCTCGGGTACCAGCTGAT-3′), human sequence for ANF (sense 5′-ATGGGCTCCTTCTCCATCAC-3′; antisense 5′-GGAGGCCCCAGCAGCCAGA-3′), and the human sequence for skeletal actin (sense 5′-AGGCCCCAGCAGCCAGA-3′; antisense 5′-GGAGGCCCCAGCAGCCAGA-3′), which recognizes both the α and β isoforms. Primers for GAPDH (sense 5′-CCTCTTGACCTCAAC-3′; antisense 5′-AGTGTTGATCATGGATACC-3′) were used for each sample as an internal control for mRNA integrity and equal loading. PCRs were carried out for GAPDH, p27\textsuperscript{KIP1}, and ANF according to previously published methods using 30 cycles of amplification, which we have shown results in the exponential phase of amplification for these genes. PCR products were carried out for skeletal actin as follows: 30 cycles of
94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 90 seconds. PCR products were separated on 16% vol/vol acrylamide gels, and the resultant gels were stained in 40 μg/mL of ethidium bromide for 5 minutes. Ethidium bromide–stained bands were viewed and photographed under UV light. mRNA samples of myocytes prepared from sham-operated (SH) and aortic-constricted (AC) rat hearts (24 hours after operation) were a gift from Dr Robert Haworth, Cardiovascular Research, St. Thomas' Hospital, London.

Restriction Enzyme Digestion of α-Skeletal Actin
PCR Fragment

The primers used for the PCR amplification of skeletal actin cDNA recognize both α and β isofoms and, therefore, they cannot be distinguished easily. However, restriction enzyme digestion with BglII permits cleavage of the α isoform into two fragments (212 and 121 bp), leaving the β isoform intact, as a 331-bp band. Ten microliters of the PCR reaction, prepared as described above, 1.5 μL of 10× buffer 7, and 1.5 μL of BglII were added together in an Eppendorf tube. The mixture was incubated at 37°C for 1 hour, and the digested products were separated on a 20% vol/vol acrylamide gel. The resultant gel was stained with 40 μg/mL ethidium bromide for 5 minutes. Ethidium bromide–stained bands were viewed and photographed under UV light.

Immunocytochemistry

Whole ventricular tissues from adult p27KIP1+/+ and p27KIP1 knockout mice were snap-frozen in liquid N2 and stored at −70°C. Sequential cryosections (4 μm) were cut at −20°C. Pretreatment of sections and immunofluorescence detection of laminin and troponin I antibodies and PI were carried out as described previously. Three sections from each tissue were analyzed by two independent assessors, and three different tissues were used for each age group.

Protein Determination
Protein concentrations in cell preparations were determined by the method of Bradford using BSA type V as a standard.

Statistical Analysis

All autoradiographs were scanned by laser densitometry (Ultrascan XL, LKB). All arbitrary values were log-transformed and were analyzed by one-way ANOVA. Data were subsequently tested for statistical significance (all pairwise comparisons) using the Student-Newman-Keuls test, and values of P<0.05 were considered to be significant.

Results

Effects of p27KIP1 Deletion on Heart Weight

The hearts of homozygous control (p27KIP1+/+), heterozygous p27KIP1 knockout (p27KIP1+/-), and homozygous p27KIP1 knockout (p27KIP1−/−) mice (5 per group) were weighed independently to determine the effect of the loss of p27KIP1 on the wet weight of the heart. In accordance with previous studies, the loss of p27KIP1 resulted in a significant increase in the size and weight of the heart in all mice studied. Thus, Figure 1 shows that by 21 days of age, the wet weights of both the p27KIP1+/− and p27KIP1−/− knockout hearts were increased significantly by >15% and by >40% respectively, compared with p27KIP1+/+ hearts. This increased size of the hearts in p27KIP1+/− and p27KIP1−/− mice was observed at all ages studied (Figure 1) and was shown to be significant from 2 days of age onward in p27KIP1−/− mice when compared with p27KIP1+/+ mice (n=3, P<0.05). As reported previously, p27KIP1+/− mice showed an intermediate enlargement of the heart, which was increased significantly from 6 days of age onward when compared with p27KIP1+/+ mice.
nuclei, and troponin I (Figure 3B and 3D) enabled us to demonstrate that the observed increase in cell number and smaller cell size in adult p27KIP1/12 hearts (Figure 2C) was occurring in the myocyte fraction of the myocardium.

**Effects of p27KIP1 Deletion on the Cell Cycle Profile of Neonatal Mouse Cardiac Myocytes**

To further substantiate that the observed increase in cell number in the p27KIP1 knockout mouse heart was due, at least in part, to an increase in the proliferation of the cardiac myocyte population, we compared the cell cycle profiles of myocytes prepared from p27KIP111, p27KIP112, and p27KIP122 hearts at different ages of postnatal development. Figure 4 shows the cell cycle profiles of p27KIP1+/+, p27KIP1+/−, and p27KIP1−/− myocytes prepared from the hearts of mice aged 1, 2, 3, 4, and 6 days old. Thus, the percentage of S phase cells (DNA synthesizing cells) in myocytes isolated from the hearts of 1-, 2-, 3-, and 4-day-old p27KIP122 mice was shown to be significantly higher than in cells isolated from p27KIP111 hearts, although similar results were observed by 6 days of age (Figure 4). Concomitant with the increase in the number of S phase cells, the percentage of G0/G1 phase cells decreased significantly in myocytes obtained from the hearts of 1-, 2-, 3-, and 4-day-old p27KIP1−/− mice when compared with p27KIP1+/+ myocytes, although levels returned to those present in p27KIP1+/+ myocytes by 6 days of age (Figure 4). Interestingly, myocytes obtained from the hearts of p27KIP1+/− mice at all of the developmental ages tested showed intermediate changes in cell cycle profile (Figure 4) when compared with the cell cycle profiles of myocytes prepared from p27KIP1−/− and p27KIP1+/+ mouse hearts.

**Expressions of Myocyte Differentiation Markers in Whole Ventricular Tissue From p27KIP1 Knockout Mice**

Given that the loss of p27KIP1 results in a prolonged proliferation of cardiac myocytes (observed as a significant increase in cell number and smaller cell size [Figures 2 and 4]), this suggests that the loss of p27KIP1 results in a less well-differentiated phenotype in myocytes present in p27KIP1−/− mouse hearts compared with myocytes in p27KIP1+/+ hearts. To test this hypothesis, we determined the effects of p27KIP1 deletion on the mRNA expressions of ANF and α-skeletal actin, which are genes expressed specifically during the embryonic stages of cardiac development such that their expressions are subsequently downregulated during normal myocyte maturation and differentiation.34–36 However, these genes are reexpressed during the development of pressure overload–induced left ventricular hypertrophy.22,34–36 mRNA samples from the murine NIH3T3 fibroblast cell line, which expresses p27KIP1 and GAPDH at detectable levels,22 and from SH and AC adult rat myocytes, which we previously have shown express ANF,22 were used as positive controls for each RT-PCR reaction. In contrast to GAPDH expression, which remained at constant levels in all hearts and served as an internal control for mRNA
loading (Figure 5A), low levels of p27KIP1 mRNA were found in 6-day-old p27KIP1+/+ whole ventricular tissue, with higher levels in adult p27KIP1+/+ ventricular tissue. This is consistent with our previous data showing the differential expression of p27KIP1 mRNA and protein during development of rat cardiac myocytes.9,18 p27KIP1 mRNA was neither detectable in whole ventricular tissue obtained from 6-day-old nor adult p27KIP1−/− mouse hearts but was detectable at very low levels in 6-day-old p27KIP1+/− heterozygous mouse hearts (Figure 5B). Consistent with our previous findings,22 mRNA expression of the hypertrophic marker ANF was present at higher levels in myocytes prepared from AC rat ventricles compared with low levels in myocytes from SH rat hearts. ANF mRNA was undetectable in whole ventricular tissue obtained from 6-day-old and adult p27KIP1−/− mouse hearts but was detectable at very low levels in 6-day-old p27KIP1+/− heterozygous mouse hearts (Figure 5B). Consistent with our previous findings,22 mRNA expression of the hypertrophic marker ANF was present at higher levels in myocytes prepared from AC rat ventricles compared with low levels in myocytes from SH rat hearts. ANF mRNA was undetectable in whole ventricular tissue obtained from 6-day-old and adult p27KIP1+/+ mouse hearts and from 6-day-old p27KIP1+/− mouse hearts but was detectable in 6-day-old and adult p27KIP1−/− whole ventricular tissue (Figure 5C). The expression of total (α and β isoforms) skeletal actin was shown to be unchanged in all mouse whole ventricular samples (Figure 5D).

Protein Expressions of Cell Cycle Regulatory Molecules in p27KIP1 Knockout Mouse Whole Ventricular Tissue

p21CIP1 and p27KIP1

Figures 6A-a and 6A-b show the expressions of p21CIP1 and p27KIP1 proteins, respectively, in p27KIP1+/+ and p27KIP1−/− whole ventricular tissues obtained from mice aged 1, 2, 3, and 4 days of postnatal development. p27KIP1 protein was undetectable at all ages in p27KIP1−/− whole ventricular tissue but was clearly demonstrable at constant levels in whole ventricular tissue obtained from adult p27KIP1+/+ mice (Figure 6A-b). In contrast, p21CIP1 protein expression was detectable at similar levels in whole ventricular tissue obtained from all ages of p27KIP1+/+ and p27KIP1−/− whole ventricular tissue obtained from mice aged 1, 2, 3, and 4 days of postnatal development. p27KIP1 protein was undetectable at all ages in p27KIP1−/− whole ventricular tissue but was clearly demonstrable at constant levels in whole ventricular tissue obtained from adult p27KIP1+/+ mice compared with tissue obtained from 6-day-old mice (Figure 6B and Reference 37). As predicted, p27KIP1−/− mouse hearts failed to demonstrate p27KIP1 protein expression whereas p21CIP1 showed a similar pattern of expression to that observed in p27KIP1+/+ samples (Figure 6B and Reference 37). The expressions of p21CIP1 and p27KIP1 in wild-type and p27KIP1−/− hearts were quantified by densitometric scanning of autoradiographs obtained from three separate hearts. Results were normalized individually to
Figure 4. Effect of p27KIP1 deletion on the cell cycle profiles of cardiac myocytes. Graphs show the percentage of p27KIP1+/+, p27KIP1+/−, and p27KIP1−/− myocytes in the G1/G0 (upper graph) and S phases (lower graph) of the cell cycle. 1, 2, 3, 4, and 6 indicate x-day-old neonatal cardiac myocytes; ND, not determined. *P<0.05 compared with myocytes prepared from age-matched p27KIP1+/+ mouse hearts (n=3).

CDKs

Figures 7A-a through 7A-d show the protein expressions of CDC2, CDK2, CDK4, and CDK6, respectively, in whole ventricular tissues obtained from p27KIP1−/− mice aged 1, 2, 3, and 4 days of postnatal development. The results clearly demonstrate that the expressions of both CDC2 and CDK2 were elevated significantly (n=3, P<0.05) in p27KIP1−/− hearts at 1 day and 2 days of age compared with age-matched p27KIP1+/+ hearts (Figure 7A-a and 7A-b); levels subsequently returned to control (p27KIP1+/+) levels by 3 days of postnatal development. The levels of CDK4 expression in ventricular tissue obtained from 1-day-old to 3-day-old p27KIP1−/− hearts were not significantly different to that found in p27KIP1+/+ ventricular tissue (Figure 7A-c). In contrast to CDC2, CDK2, and CDK4, the expression of CDK6 was downregulated significantly in whole ventricular tissues from 1-day-old to 3-day-old p27KIP1−/− mice (n=3, P<0.05) compared with age-matched p27KIP1+/+ hearts (Figure 7A-d). In whole ventricular tissue obtained from 6-day-old and adult p27KIP1−/− mice, the expressions of CD2, CDK2, CDK4, and CDK6 were maintained at similar levels to those found in age-matched p27KIP1+/+ mice (Figure 7B). The changes in expressions of the CDKs in the p27KIP1−/− mouse hearts were quantified by densitometric scanning of resultant autoradiographs obtained from three separate hearts. Results were normalized individually to the 1-day-old p27KIP1+/+ sample, which was expressed as a densitometry index value of 1 in Figure 6C. Thus, there was no significant change in the level of p21CIP1 in the p27KIP1−/− hearts at any stage of development. Therefore, the loss of p27KIP1 does not appear to be compensated for by the upregulation of the CDKI molecule p21CIP1. Moreover, and consistent with a recent report,21 we were unable to detect the protein expression of other CDKI molecules, including p57KIP2 or any member of the INK4 family, in whole ventricular tissue obtained from mice at any stage of development (data not shown).

Figure 5. Expressions of specific differentiation markers in p27KIP1 knockout hearts. Panels A, B, C, D, and E show expressions of GAPDH, p27KIP1, ANF, skeletal actin, and skeletal actin digested with BglII, respectively. QNIH indicates quiescent NIH3T3 fibroblasts; SH, myocytes obtained from adult rat hearts that had undergone sham operation; AC, myocytes obtained from adult rat hearts that had undergone aortic constriction; d6, 6-day-old; Ad, adult; +/+ , wild-type p27KIP1+/+ mice; +/−, p27KIP1 heterozygous knockout mice; and −/−, p27KIP1 homozygous knockout mice.

Cyclins and PCNA

Figures 8A-a through 8A-d show the expressions of cyclins A, E, D2, and D3, respectively, in whole ventricular tissues obtained from p27KIP1+/+ and p27KIP1−/− mice aged 1, 2, 3, and 4 days of postnatal development. The expressions of cyclins A and E were upregulated significantly (n=3, P<0.05) in whole ventricles obtained from 1-day-old to 3-day-old p27KIP1−/− mice compared with their age-matched wild-type controls (Figure 8A-a and 8A-b). In contrast, the
expressions of cyclins D2 and D3 were downregulated significantly (n=3, P<0.05) in hearts from all p27 KIP1 /−/− mice compared with age-matched p27 KIP1+/+ hearts (Figure 8A-c and 8A-d). In addition, by 6 days of postnatal development, cyclin A expression in p27KIP1 knockout hearts returned to levels similar to that detectable in p27 KIP1+/+ whole ventricular tissue, whereas cyclin E protein remained elevated in tissue obtained from 6-day-old neonatal whole ventricular tissue; Ad, adult whole ventricular tissue; +/+ , wild-type p27KIP1+/+ mice; +/− , p27KIP1 heterozygous knockout mice; and −/−, p27KIP1 homozygous knockout mice.

Figure 6. Expressions of p21CIP1 and p27KIP1 proteins in p27KIP1 knockout mouse whole ventricular tissues. A and B, Expressions of p21CIP1 and p27KIP1 proteins in whole ventricular tissue obtained from 1-day-old to 4-day-old mice and 6-day-old and adult mice, respectively. C, Bar graph of the changes in protein expressions of p21CIP1 and p27KIP1 in p27 KIP1−/− hearts. Results were normalized to expression of the same protein in 1-day-old wild-type hearts. Values are mean±SD. *P<0.05 compared with age-matched wild-type controls. 1d, 2d, 3d, 4d, and 6d indicate x-day-old neonatal whole ventricular tissue; Ad, adult whole ventricular tissue; 1/1, wild-type p27KIP1+/+ mice; 1/2, p27KIP1 heterozygous knockout mice; and 2/2, p27KIP1 homozygous knockout mice.

expressions of cyclins D2 and D3 were downregulated significantly (n=3, P<0.05) in hearts from all p27KIP1−/− mice compared with age-matched p27KIP1+/+ hearts (Figure 8A-c and 8A-d). In addition, by 6 days of postnatal development, cyclin A expression in p27KIP1 knockout hearts returned to levels similar to that detectable in p27KIP1+/+ whole ventricular tissue, whereas cyclin E protein remained elevated in tissue obtained from 6-day-old p27KIP1−/− mice but returned to normal (p27KIP1+/+) levels by the adult stage of development (Figure 8B). In contrast, the expressions of both cyclins D2 and D3 remained significantly downregulated in p27KIP1−/− cardiac tissue at all ages, except for cyclin D3, which returned to normal levels by the adult stage of development (Figure 8B). The changes in expressions of the cyclins in the p27KIP1−/− mouse hearts were quantified by densitometric scanning of resultant autoradiographs obtained from three separate hearts. Results were normalized individually to the 1-day-old p27KIP1+/+ sample, which was expressed as a densitometry index value of 1 in Figure 8C.

Thus, a significant increase in the protein levels of cyclins A and E (1 day to 3 days) and a significant decrease in the protein levels of cyclins D2 and D3 (1 day to 6 days [D3]; 1 day to 36 days [D2]) was observed in p27KIP1−/− hearts compared with wild-type hearts (P<0.05). Consistent with
our other results, an intermediate change in the expression of each cyclin was seen in ventricular tissue obtained from age-matched p27KIP1 knockout mouse hearts, A and B. Expressions of cyclins A, E, D2, and D3 in whole ventricular tissues obtained from 1-day-old to 4-day-old mice and 6-day-old and adult mice, respectively. C, Bar graph of the changes in protein expressions of the cyclins in p27KIP1 knockout hearts. Results were normalized to expression of the same protein in 1-day-old wild-type hearts. Values are mean±SD. *P<0.05 compared with age-matched wild-type controls. SS NIH indicates serum-stimulated NIH3T3 fibroblasts; 1d, 2d, 3d, 4d, and 6d, x-day-old neonatal whole ventricular tissue; Ad, adult whole ventricular tissue; +/+ wild-type p27KIP1+/+ mice; +/-, p27KIP1 heterozygous knockout mice; −/−, p27KIP1 homozygous knockout mouse.

Figure 9A shows the expression of the proliferation marker PCNA in whole ventricular tissues obtained from p27KIP1+/+ and p27KIP1−/− mice aged 1, 2, 3, and 4 days of postnatal development such that expression of this protein was elevated significantly in whole ventricular tissue from 1-day-old to 3-day-old p27KIP1−/− mouse hearts, relative to levels expressed in age-matched p27KIP1+/+ tissues (>2-fold, n=3, P<0.05). Levels subsequently returned to control (p27KIP1+/+) levels in whole ventricular tissues obtained from p27KIP1−/− mice at 4 days of age (Figure 9A). Interestingly, at 6 days of postnatal development, the expression of PCNA was significantly elevated in p27KIP1−/− whole ventricular tissue compared with p27KIP1+/+ samples (Figure 9B). This elevated level of expression subsequently returned to p27KIP1+/+ levels by the adult stage of development (Figure 9B). The change in expression of PCNA in the p27KIP1−/− mouse hearts was quantified by densitometric scanning of resultant autoradiographs obtained from three separate hearts. Results were normalized individually to the 1-day-old p27KIP1+/+ sample, which was expressed as a densitometry index value of 1 in Figure 9C. Thus, a significant increase in the protein level of PCNA (1 day to 3 days and 6 days) was observed in p27KIP1−/− hearts compared with wild-type hearts (P<0.05). Age-matched p27KIP1+/+ mouse whole ventricles displayed an intermediate level of PCNA expression compared with levels in p27KIP1+/+ and p27KIP1−/− mouse hearts (Figure 9B and data not shown).

**Discussion**

The ability of the mouse cardiac myocyte to undergo hyperplasia previously has been shown to be lost at birth, although
the mechanism(s) underlying this cell cycle withdrawal still remains to be determined. In the present study, we have made use of the p27KIP1 knockout mouse and have investigated, for the first time, the effects of p27KIP1 deletion on myocyte proliferation in the mouse ventricle. In addition, we have investigated the effects of loss of p27KIP1 on the cell cycle profiles of neonatal mouse cardiac myocytes and on the expressions of certain other cell cycle regulatory proteins that have been proposed to play a role in the withdrawal of the cardiac myocyte from the cell cycle.

In accordance with previous studies, we have shown that loss of p27KIP1 results in a significant enlargement in the size and weight of the mouse heart (Figure 1). Furthermore, deletion of a single allele in the p27KIP1 gene (p27KIP1+/− mouse) led to intermediate levels of cardiac growth compared with normal wild-type (p27KIP1+/+) and homozygous knockout (p27KIP1−/−) mice, thereby suggesting a dose-dependent effect of p27KIP1 on cardiac growth. Multiple organ enlargement previously has been reported in the p27KIP1 knockout mouse, and this has been shown, in certain organs other than the heart, to be due to cellular hyperplasia, rather than cellular hypertrophy. Because the heart consists of both myocyte and nonmyocyte populations, we wished to determine whether the loss of p27KIP1 had any effect on the ability of the cardiac myocyte to proliferate. Using immunocytochemical staining of whole ventricular cryosections, we have shown that the number of cardiac myocytes increases significantly by 2- to 3-fold in the adult p27KIP1−/− mouse heart (n=3, P<0.05) compared with wild-type adult hearts (Figures 2 and 3). Furthermore, the increase in cell number was not associated with an increase in myocyte cell size (hypertrophy) in p27KIP1−/− hearts, whereas myocytes in p27KIP1+/+ hearts exhibited hypertrophy (Figure 2). Indeed, the increase in cell number but smaller cell size is in accordance with results reported for other tissues lacking p27KIP1. We propose, therefore, that the loss of p27KIP1 results in additional rounds of proliferation of the cardiac myocyte population, coupled with a perturbation of myocyte hypertrophy and terminal differentiation, as reported previously in other cell types lacking p27KIP1. Furthermore, the results of our study suggest that p27KIP1 is a major component of the mechanism that determines when cells stop dividing and differentiate. However, the fact that p27KIP1−/− myocytes appear to undergo only one to two additional cell divisions before they exit the cell cycle and differentiate (see above and References 27 and 38) suggests that p27KIP1 is only one component of the timer mechanism for cell cycle arrest and is not sufficient on its own for cells to exit the cell cycle. Interestingly, during early development of the p27KIP1−/− heart, we have shown that there are significant changes in the expressions of certain cyclins and CDKs, with little change in the expressions of CDK1 molecules (Figures 6 through 8). This suggests that the loss of certain positive regulators may provide an important component of the cell cycle stopping mechanism in cardiac myocytes, as has been suggested previously in other cell types.

Thus, we have shown that the protein expressions of the G1/S and G2/M phase–acting cyclins and CDKs (ie, cyclins A and E, CDC2 and CDK2) were upregulated significantly during the proposed extended rounds of myocyte division in the p27KIP1−/− mouse heart (Figures 7 and 8). In contrast, the protein expressions of the G1 phase–acting cyclins and CDKs (ie, D-type cyclins and CDK6) were downregulated significantly during the early neonatal developmental period in p27KIP1−/− mouse hearts (Figures 7 and 8), despite a significant increase in the number of S phase cells (Figure 4). The reason for the decrease in the expressions of the G1 phase–acting molecules in the p27KIP1−/− heart coupled with an increase in the number of S phase myocytes remains unknown. However, it is possible that the loss of p27KIP1 compensates for the requirement for any increase in G1 phase–acting molecules, and the corresponding increase in the expressions of the G1/S and G2/M phase–acting molecules then enables increased proliferation of the p27KIP1−/− cardiac myocyte. We previously have shown that the expressions and activities of the G1/S and G2/M phase–acting cyclins and CDKs are downregulated significantly during the switch from myocyte hyperplasia to hypertrophy in the rat, whereas the G1 phase–acting cyclins and CDKs are upregulated. These results indicate that the G1/S and G2/M phase–acting molecules may be critical for the switch from myocyte hyperplasia to hypertrophy and that the G1 phase–acting molecules may be important for the processes of hypertrophy and binucleation. Therefore, the observed changes in the protein expressions of the positive regulators of the cell cycle could potentially contribute to the increase in myocyte cell number and smaller cell size observed in the p27KIP1−/− mice.

Interestingly, studies in Drosophila have shown that the inactivation of CDC2 in the wing disk during pupal development (where only one cell divisions occur before terminal differentiation) results in the formation of larger cells, but the final wing is normal in size, with fewer cells. However, the inactivation of CDC2 in larval Drosophila, at early stages of development (where cell numbers are increasing exponentially), results in a smaller wing size due to a reduced cell number. The increased proliferation of the cardiac myocyte, associated with the lack of hypertrophy, indicates that p27KIP1 has important roles in the control of both cell division and cell growth. CDC2, on the other hand, would appear to have a critical role in regulating cell division only. Therefore, the results of our study, and that of others, suggest that the inactivation of cell cycle–dependent molecules, at a time when cells are actively proliferating, results in a significant effect on the overall organ size. In contrast, it would seem that the inactivation of critical cell cycle genes, at a time when cells are about to withdraw from the cell cycle, results in little effect on the final organ size.

It is interesting to note that the cardiac phenotype observed in the p27KIP1−/− heart is similar to that observed in c-myc overexpressing mice (see above and References 43 and 44). The transcription factor Myc is known to mediate its mitogenic activity by upregulating the expression of a number of cell cycle regulatory molecules (eg, cyclin A and E2F-1). It should also be noted that p21CIP1 and p27KIP1 activity can be sequestered by Myc-induced proteins. Therefore, given the link between Myc and the cell cycle, it is not surprising that the cardiac phenotypes of the c-myc overexpressing and p27KIP1−/− mice are very similar.
Given that p27KIP1 has been shown to play a critical role in controlling the cell cycle exit of numerous cell types, it is likely that in addition to the increased proliferation of the cardiac myocyte fraction of the heart, an increased proliferation of the nonmyocyte fraction also contributes to the enhanced size of the p27KIP1 knockout mouse heart. In addition, other factors (eg, environmental stresses) have been shown to affect the growth potential of the cardiac myocyte, and, hence, we cannot completely exclude the possibility that the cardiac phenotype observed in the p27KIP1/−/− mice is not contributed to by factors other than p27 deficiency. However, we are confident that the increase in myocyte number in p27KIP1/−/− mice largely occurs as a result of alterations in the integral cell cycle regulatory response (cell autologous effect) rather than an environmental response for the following reasons. (1) Homozygous, heterozygous, and wild-type animals were born into litters of similar sizes (mean ± SD of the number of pups per litter: homozygous/heterozygous = 8.3 ± 1.3, wild type = 8.6 ± 1.0) and therefore were subjected to similar environmental factors. (2) In contrast to the results reported by Bai et al, where the environmental effects persisted into adulthood, the change in phenotype resulting from p27KIP1 deficiency did not persist into adulthood (Figures 4 through 9).

We have shown that there is a significant G1/G phase cell cycle blockade at the time when the normal mouse cardiac myocyte loses its ability to proliferate (Figure 4). Interestingly, in wild-type myocytes, the percentage of cells synthesizing DNA dropped to its lowest level in hearts at 2 days and 3 days of age, after which the number of S phase cells increased slightly, concomitant with a reduction in the number of G1/G0 phase cells (Figure 4). This is in accordance with the results of Soonpaa et al who showed that the decrease in the number of S phase myocytes at 2 days and 3 days of age in the mouse correlates with the decrease in the proliferation of the myocyte. Furthermore, the increase in the number of S phase cells at 4 days of age was shown to correlate with the onset of myocyte binucleation. In contrast, myocytes obtained from p27KIP1/−/− mouse hearts displayed a significant increase in the number of S phase cells at this early postnatal age, concomitant with a significant decrease in the number of G1/G0 phase cells (Figure 4). Thus, the maintenance of high levels of DNA synthesis in the p27KIP1 knockout myocytes strongly suggests that the increased DNA synthesis results in the ability of the cardiac myocyte to extend its natural number of proliferation cycles before its withdrawal from the cell cycle and its subsequent differentiation. Furthermore, the elevated level of expression of PCNA in hearts obtained from 1-day-old to 3-day-old p27KIP1/−/− mice correlated well with the observed increase in cellular proliferation during early p27KIP1/−/− heart development (Figure 9). After returning to control (p27KIP1/+/+) levels at 4 days of age, the second increase in PCNA expression in the whole ventricle obtained from 6-day-old p27KIP1/−/− mice (Figure 9) is similar to that observed at 4 days of age in the wild-type mouse heart (Figure 9 and Reference 3), an age that correlates with an increase in DNA synthesis and myocyte binucleation. This suggests that the p27KIP1/−/− heart, the onset of myocyte binucleation is delayed by ≈2 days, which correlates with the extended natural number of cell divisions and perturbation of hypertrophy in the p27KIP1/−/− heart.

A delay in the onset of cardiac myocyte hypertrophy and differentiation also occurs in p27KIP1/−/− mice, such that the mRNA expressions of the embryonic genes ANF and α-skeletal actin, which normally are downregulated shortly after birth, are detectable at significant levels in whole ventricular tissues obtained from 6-day-old p27KIP1/−/− mouse hearts (Figure 5). Therefore, we have shown by a combination of techniques that mouse cardiac myocytes lacking p27KIP1 are able to undergo additional rounds of cell division after birth, delaying their terminal differentiation.

In summary, we have shown for the first time that the loss of the CDKI molecule p27KIP1 in the mouse heart results in a prolonged proliferation of cardiac myocytes and a perturbation of cardiac myocyte hypertrophy and differentiation. It is possible that the combination of the loss of p27KIP1 and the concomitant increase in the expressions of G1/S and G2/M phase–acting molecules is critical for prolonging myocyte proliferation for a finite number of additional divisions in p27KIP1 knockout mouse hearts. It also is possible that the observed decrease in the expressions of G1 phase–acting molecules may contribute to the perturbation of myocyte hypertrophy and differentiation seen in p27KIP1 knockout mouse hearts, considering the fact that the expressions and activities of these molecules are upregulated during hypertrophic growth of the adult rat myocyte. The use of knockout mouse models lacking specific cell cycle regulatory molecules will enable us to fully dissect the role of specific cell cycle proteins in controlling the switch from myocyte hyperplasia to hypertrophy and may help us to identify those molecules that are critical for myocyte cell cycle arrest. Specific targeting of such molecules in the future may enable us to reinitialize, in a controlled manner, cell division of healthy myocytes that surround an infarcted zone.

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Altered Expression of Cell Cycle Proteins and Prolonged Duration of Cardiac Myocyte Hyperplasia in p27 KIP1 Knockout Mice
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